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Increased maturation of iPSC-derived neurons in a hydrogel-based 3D culture

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Abstract: BACKGROUND Induced pluripotent stem cells (iPSCs) can be differentiated into virtually every desired cell type, offering significant potential for modeling human diseases in vitro. A disadvantage is that iPSC-derived cells represent an immature, which presents a major limitation for modeling age-related diseases such as Alzheimer's disease. Evidence suggests that culturing iPSC neurons in a 3D environment may increase neuronal maturity. However, current 3D cell culture systems are cumbersome and time-consuming. NEW METHOD We cultured iPSC-derived excitatory neurons in 3D precast hydrogel plates and compared their maturation to 2D monolayer cultures. COMPARISON WITH EXISTING METHODS In contrast to other hydrogel-based 3D culture techniques, which require full encapsulation of cells, our hydrogel allows the seeded iPSCs and iPSC neurons to simply infiltrate the gel. RESULTS iPSC-neurons grew to a depth of 500 µm into the hydrogel. Cell viability was comparable to 2D cultures over the course of three weeks, with even better neuronal survival in 3D cultures at the one-week time point. Levels of neuronal and synaptic maturation markers, namely, neural cell adhesion molecule 1 (NCAM1) and -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR2, were strongly increased in 3D cultures. Furthermore, we identified 4-repeat (4R) tau in 3D cultures, which was not detectable in 2D cultures. CONCLUSIONS We describe a simple, hydrogel-based method for 3D iPSC culture that can serve as a fast and drug-screening-compatible platform to identify new mechanisms and therapeutic targets for brain diseases. We further provided evidence for the increased maturation of iPSC neurons in a 3D microenvironment.

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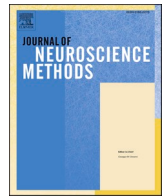


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Short communication

Increased maturation of iPSC-derived neurons in a hydrogel-based 3D culture

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ABSTRACT

Background: Induced pluripotent stem cells (iPSCs) can be differentiated into virtually every desired cell type, offering significant potential for modeling human diseases *in vitro*. A disadvantage is that iPSC-derived cells represent an immature, which presents a major limitation for modeling age-related diseases such as Alzheimer's disease. Evidence suggests that culturing iPSC neurons in a 3D environment may increase neuronal maturity. However, current 3D cell culture systems are cumbersome and time-consuming.

New method: We cultured iPSC-derived excitatory neurons in 3D precast hydrogel plates and compared their maturation to 2D monolayer cultures.

Comparison with existing methods: In contrast to other hydrogel-based 3D culture techniques, which require full encapsulation of cells, our hydrogel allows the seeded iPSCs and iPSC neurons to simply infiltrate the gel.

Results: iPSC-neurons grew to a depth of 500 μm into the hydrogel. Cell viability was comparable to 2D cultures over the course of three weeks, with even better neuronal survival in 3D cultures at the one-week time point. Levels of neuronal and synaptic maturation markers, namely, neural cell adhesion molecule 1 (NCAM1) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR2, were strongly increased in 3D cultures. Furthermore, we identified 4-repeat (4R) tau in 3D cultures, which was not detectable in 2D cultures.

Conclusions: We describe a simple, hydrogel-based method for 3D iPSC culture that can serve as a fast and drug-screening-compatible platform to identify new mechanisms and therapeutic targets for brain diseases. We further provided evidence for the increased maturation of iPSC neurons in a 3D microenvironment.

1. Introduction

Induced pluripotent stem cell (iPSC) technology has induced a paradigm shift in biomedical research, as it enables the use of human cells for disease modeling and drug discovery. Nevertheless, some limitations exist that reduce the value of iPSCs for modeling age-related diseases, such as the immature nature of differentiated iPSCs and their resemblance to fetal tissue (de Leeuw and Tackenberg, 2019). A way to circumvent the lack of maturity is to differentiate iPSCs in a 3D environment, such as hydrogel-based cultures, neurospheres, or self-organizing 3D neural tissue (Zhang et al., 2016; Raja et al., 2016;

Lancaster et al., 2017).

Hydrogels, based on substrates such as polyethylene glycol (PEG), have been used for 3D cell cultures and tissue engineering for several years (Tan et al., 2010). Human neurons and glial cells have been successfully cultured in starPEG-heparin-based hydrogels, forming complex 3D structures (Papadimitriou et al., 2018). Furthermore, elevated synaptic density was observed in iPSC-derived neurons embedded in a layered, hyaluronic acid-based hydrogel (Zhang et al., 2016). Choi et al. showed increased maturation of human NPC-derived neurons embedded in 0.3–4 mm-thick Matrigel (Choi et al., 2014). Increased maturity was evident by elevated levels of markers for neuronal and synaptic

Abbreviations: iPSC, induced pluripotent stem cell; PEG, poly(ethylene glycol); NCAM1, neural cell adhesion molecule 1; GluR2, AMPA receptor subunit 2; AMPA receptor, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AD, Alzheimer's disease.

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maturation, such as NCAM1 or NMDA-type glutamate receptor subunit NR2A. It should be noted that the NPCs used in that study were not derived from iPSCs. In mature neurons, tau protein, a major contributor to Alzheimer's disease (AD) pathology, is found in six different splice variants, depending on the presence of three or four repeat domains, named 3R tau and 4R tau, respectively. Both 3R and 4R tau are found in neurofibrillary tangles in the AD brain. However, iPSC-derived neurons cultured in 2D usually express only the fetal 3R tau isoform (ON3R) (de Leeuw and Tackenberg, 2019). This limits the use of 2D cultures for modeling AD and other tauopathies *in vitro*. Long-term 2D cultures showed the expression of 4R tau in iPSC-derived cortical neurons after 365 days (Sposito et al., 2015). In contrast, 4R tau was already detectable after 15 weeks in iPSC neurons cultured inside Matrigel-coated alginate capsules (Miguel et al., 2019).

Despite the marked improvement that these techniques offer over conventional 2D neural cultures, and current 3D protocols also show some limitations. These include poor nutrient support, a necrotic core in 3D spheres, complex and time-consuming culture conditions, low degree of reproducibility in organoids, and the requirement to encapsulate cells in hydrogels, thereby preventing later modification of the cell composition. Using a hydrogel gradient-based 3D cell culture system, we report a simple, straightforward method to increase the maturation of iPSC-derived neurons in a 3D microenvironment.

2. Materials and methods

2.1. Seeding and differentiation of iPSCs to iN cells in 2D and 3D

iPS cell line HCS1-2 was cultured on Matrigel-coated plates and characterized as previously described (Birnbaum et al., 2018). Culture and neuronal differentiation conditions were identical between 2D and 3D cell cultures. On day-1, iPSCs were dissociated with Accutase (ThermoFisher) and 45,000 iPS cells (2D) or 60,000 iPS cells (3D) were plated per well in 96-well plates (ThermoFisher) or 3DProSeed hydrogel 96-well plates (Ectica Technologies) (Zhang et al., 2017), respectively. 2D 96-well plates and 3D hydrogel plates were pre-coated with growth factor-reduced Matrigel (200 µg/mL, Corning). Cells were transduced with lentiviruses expressing rtTA and Neurogenin-2. On day 0, the medium was changed to induction medium [DMEM/F12 (ThermoFisher) with doxycycline (2 µg/mL, Sigma), 1x N2 (ThermoFisher), 1x NEAA (Sigma), BDNF (10 ng/mL, Peprotech), NT-3 (10 ng/mL, Peprotech), and laminin (200 µg/mL, Sigma)]. On day 1, the induction medium was changed and supplemented with puromycin (1 µg/mL, Sigma) for a 24 h selection period. On day 2, 45,000 primary mouse glia cells (2D) or 60,000 primary mouse glia cells (3D) were added to iN-neurobasal medium [Neurobasal-A (ThermoFisher) with 1x B27 (Gibco), 2 mM Glutamax (Gibco), 2 µM AraC (Sigma), 2 µg/mL doxycycline, 10 ng/mL BDNF, 10 ng/mL NT-3, and 200 µg/mL laminin]. Fifty percent of the medium was changed every other day until day 10, when the medium was changed to neuronal medium [MEM (ThermoFisher), 1x B27, 0.5% glucose, 0.02% NaHCO₃, 100 µg/mL transferrin (Sigma), 5% FCS, 0.5 mM L-glutamine, and 2 µM AraC]. The neuronal medium was changed twice a week until the cells were assayed on day 21. 3DProSeed hydrogel plates with different bulk modifications, i.e. addition of PLL (20–50 µg/mL), laminin (20–500 µg/mL) or combinations of both, were obtained from Ectica Technologies and tested for viability of the seeded cells.

2.2. Primary glia preparation and maintenance

Glial cells were prepared from P1 wild-type CD1 mouse pups. The brain was extracted, meninges were removed, and the forebrain was isolated. Forebrains were digested in 10 U/mL papain (Worthington) for 30 min and the cells were dissociated by pipetting. Cells were plated in DMEM with 10% fetal calf serum (FCS) in t75 flasks. Glia were split 1:3 with trypsin (ThermoFisher) at 80% confluence and were used for co-cultures between passages 2 and 4.

2.3. Imaging

To follow neuronal differentiation over time, iPSCs were transduced with an EGFP-expressing lentivirus (Birnbaum et al., 2018). EGFP fluorescence was imaged using a Leica DMIL LED fluorescence microscope equipped with a 10x objective (Fig. 1B). To analyze the depth the iN cells reached in 3D, cultures were fixed in 4% PFA after 21 days and imaged using a LEICA SP5 confocal laser scanning microscope equipped with a long distance 20x objective. Stacks of 10 µm were acquired through the entire thickness of the gel, and 3D reconstructions were generated using Fiji open-source image processing software (Fig. 1C). In-depth growth was calculated by measuring the distance of the EGFP signal in the z-direction.

2.4. Cell viability analysis

Cell viability analysis was performed by labeling dead or dying cells with 0.5 µM ethidium homodimer 1 (EthD-1, Molecular Probes) and 5 µg/mL Hoechst (ThermoFisher) according to the manufacturer's recommendations and by fluorescence live imaging. Neuronal viability was determined by counting EthD-1 positive puncta that co-localized with EGFP-expressing neuronal cells. Glial cell viability was analyzed by determining non-neuronal EthD-1 positive punctae per total number of Hoechst-positive cells, subtracted by the total number of neurons. Only EthD-1 positive punctae were analyzed, which colocalized with Hoechst to avoid unspecific binding of EthD-1 on the plates.

2.5. Lysates and western blot analysis

To obtain 3D cell lysates, the hydrogel was dissolved in trypsin/EDTA for 20 min at 37 °C. MEM + 10%FCS was added to inactivate the trypsin, and 16 3D wells were pooled and transferred into a tube. Cells were harvested by centrifugation at 200 G for 4 min, and cell pellets were resuspended in RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 0.5% SDS, 0.5% sodium deoxycholate, 2 mM EDTA) supplemented with protease inhibitor (Roche), frozen, and stored at -20 °C for further analysis. Lysates of 2D plates were performed identically by omitting the initial hydrogel dissolution step. Western blotting was performed using infrared fluorescent dye (IRDye)-coupled antibody antibodies and detected with an Odyssey CLx Imager (Licor). Image analysis was performed using ImageJ software. Signals were normalized to human GAPDH, probed using the same blot.

2.6. Antibodies used in this study

2.6.1. Western blot

Anti-human NCAM1 antibody (R&D Systems, 1:2000), anti GluR2 (ThermoFisher, 1:250), anti 4R tau (Millipore, 1:1000), and anti-human GAPDH (Abcam, 1:1000). Respective IRDye-coupled secondary antibodies were used at a 1:5000 dilution.

2.6.2. Immunocytochemistry

Anti Tra-1-60 antibody (Millipore, 1:100) and anti-Nanog (R&D Systems, 1:100). Donkey anti-mouse (Alexa488, Jackson) and donkey anti-goat (Cy3, Jackson) secondary antibodies were used at a 1:250 dilution.

2.7. Statistical analysis

Data are presented as mean ± standard deviation (SD). All data points (n) are plotted individually in each bar graph. Statistical analysis was performed with GraphPad Prism 7.01 software using the Shapiro-Wilk test for normal distribution. For comparison of multiple groups, the Kruskal-Wallis test followed by Dunn's multiple comparisons test or one-way ANOVA with Bonferroni's multiple comparisons test was performed. For comparison of two groups, an unpaired two-tailed *t*-test was

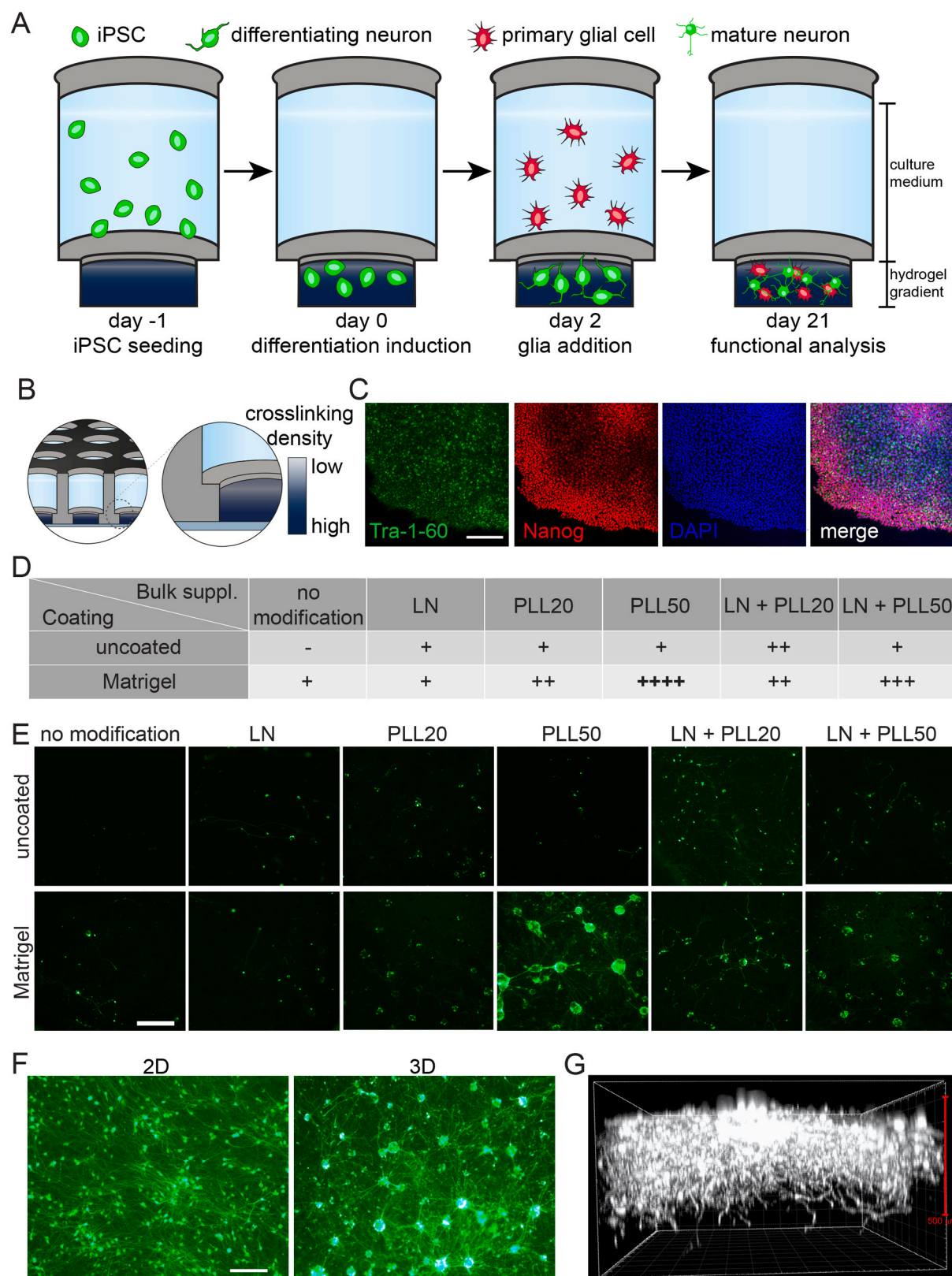


Fig. 1. Induced pluripotent stem cell (iPSC)-derived neurons cultured in 3D hydrogel plates. (A) Illustration and timeline of the experimental paradigm. (B) Schematic representation demonstrating the structure of the 96-well pre-casted hydrogel cell culture plate. (C) Representative images of the iPSC cell line used in this study. iPSCs expressed the pluripotency markers Nanog and Tra-1-60. (D) Table illustrating the analyzed cell culture conditions of 3D hydrogels at DIV21. Wells were either coated with Matrigel or uncoated. Hydrogel composition was modified by incorporating laminin and/or poly-L-lysine. (E) Representative confocal images of EGFP-expressing neurons at DIV21, cultured under the conditions mentioned in (D). (F) Representative live fluorescence images of EGFP-expressing neurons at DIV21. (G) Three-dimensional reconstruction of a confocal image stack showing that neurons grow to a depth of approximately 500 μm into the hydrogel at DIV21. Neurons were visualized by EGFP expression. LN: laminin; PLL50: poly-L-lysine 50 $\mu\text{g/mL}$. Scale bars: 100 μm .

used. All values and statistical analyses of the data in this manuscript are displayed in Suppl. Table 1.

3. Results

To establish an iPS-based 3D cell culture system, iPSCs were plated on synthetic and optically transparent hydrogels, precast in 96-well imaging plates featuring an in-depth surface density gradient (Fig. 1A and B) (3DProSeed, Ectica Technologies AG) as well as on conventional 2D 96-well plates for comparison. Previously, we and others have shown that lentiviral overexpression of Neurogenin2 in iPSCs yields a pure culture of excitatory neurons, called iN cells. These iN cells are functional after 3 weeks but show a low degree of maturity, as evidenced by low levels of certain synaptic proteins or by the lack of 4R tau expression, even in the presence of primary mouse glia to support survival and neuronal functions (Birnbbaum et al., 2018; Zhang et al., 2013). This differentiation technique requires sequential seeding, as primary mouse glial cells need to be added in a 1:1 ratio to neurons in 2D and 3D, 3 d after initial iPSC plating, as previously described (Birnbbaum et al., 2018) (Fig. 1A). Therefore, this technique is not compatible with the currently available 3D culture systems, which do not allow sequential seeding of cells. In our study, we used a well-established iPS cell line (HCS1-2; Fig. 1C), which originated from a healthy control donor (Birnbbaum et al., 2018). When iPSCs were seeded on pure PEG-based hydrogels, no surviving cells were observed (Fig. 1D and E). Therefore, we tested different coatings and modifications of the hydrogel with adhesion molecules, such as laminin (LN) and poly-lysine (PLL), for survival and homogenous distribution of cells, which was evaluated by visual inspection of confocal images as follows: –: No visible surviving cells. +: Low number of surviving cells, no visible neural networks. ++: Higher

cell number, but still lacking neural network formation and presence of dystrophic neurites. +++: Normal neurites without signs of dystrophy. ++++: High cell number with normal neuronal morphology and dense neural network formation. The incorporation of LN and PLL at different concentrations in the gel with a Matrigel coating significantly improved cell survival as well as neural outgrowth and complexity (Fig. 1D and E). We found that hydrogel supplementation with 50 µg/mL PLL (plate Nr.: ECT.PS2), combined with Matrigel coating, gave the best results; therefore, we used these conditions for further experiments.

To follow neuronal differentiation over time, cells were transduced with an EGFP-expressing lentivirus. After 3 weeks of culture, EGFP-expressing iN cells showed typical neuronal morphology in 2D and 3D, with some cell clumping in 3D (Fig. 1F). The vertically increasing cross-linking density gradient enabled cells to grow to a depth of approximately 500 µm into the hydrogel, as visualized by 3D reconstruction of confocal z-stacks (Fig. 1G, Suppl. Video 1).

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Next, we measured cell viability in 2D and 3D cultures by labeling dead or dying cells with ethidium homodimer 1 (EthD-1) (Fig. 2A). After one week of culture, fewer neurons in 3D were positive for EthD-1 than in 2D, indicating higher viability after seeding and induction of differentiation. Neuronal EthD-1 positivity after two and three weeks of culture was similar between 2D and 3D cultures (Fig. 2A, left). No differences were observed in the viability of sequentially seeded glial cells over the course of three weeks (Fig. 2A, right).

To assess the potential increase in neuronal maturation, we analyzed the levels of marker proteins for neuronal and synaptic maturity using western blotting (Fig. 2B–E). We observed a significantly higher expression of NCAM1 in 3D cultures than in 2D cultures ($p < 0.0001$).

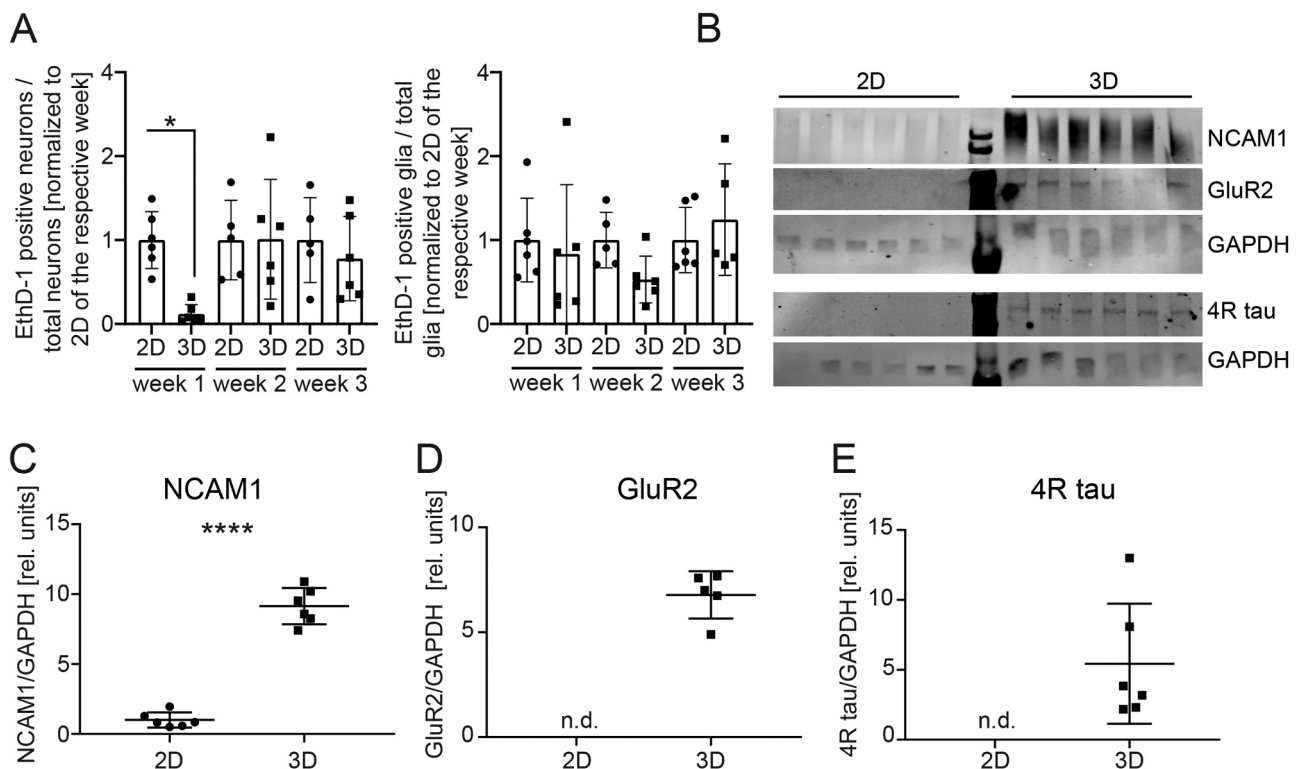


Fig. 2. Analysis of neuronal viability and maturation in 3D hydrogel culture compared to 2D cultures. (A) EthD-1 assay showing dead or dying neurons (left) and glial cells (right) in 2D and 3D. (B) Representative western blots of lysates of 2D and 3D cultures. (C–E) Quantification of western blot band intensities, corrected for the respective loading control (GAPDH), showing increased levels of NCAM1, GluR2 and 4R tau in 3D compared to 2D cultures. Data are presented as mean ± standard deviation (SD). All datapoints (n-numbers) are plotted individually in each bar graph. Statistical analysis was performed using GraphPad Prism 7.01 software. Shapiro-Wilk test was applied to test for normal distribution. For comparison of multiple groups one-way ANOVA with Bonferroni's multiple comparisons test was performed (2A, left) or Kruskal-Wallis test followed by Dunn's multiple comparisons (2A, right). For statistical comparison of two groups (2C) unpaired, two-tailed *t*-test was used (* $p < 0.05$; **** $p < 0.0001$).

Furthermore, we detected the expression of AMPA receptor subunit GluR2 and 4R tau in 3D cultures, while both proteins were below the detection limit in 2D cultures.

4. Discussion

We established a 3D cell culture system for iPS-derived neuronal cultures with an in-depth growth of approximately 500 μm . In other studies, the three-dimensional expansion usually ranged from 100 to 500 μm (Zhang et al., 2016; Choi et al., 2014; Miguel et al., 2019), but has also reached approximately 1 mm (Zhang et al., 2016; Choi et al., 2014), depending on the cell type.

Current 3D culture techniques, such as self-organizing organoid (Lancaster et al., 2017), neural tissue (Raja et al., 2016), and hydrogel (PEG-or Matrigel)-based methods (Papadimitriou et al., 2018; Choi et al., 2014) ultimately encapsulate the cells and do not allow for subsequent manipulation of cell number or cell type composition. In contrast, the density gradient in our hydrogels enabled subsequent changes in cell composition, thereby facilitating more complex co-culture systems. Furthermore, while our iN cell culture still requires Matrigel coating for initial iPSC seeding and adherence, other cell types may be cultured without Matrigel, benefitting from a defined hydrogel composition and leading to improved reproducibility. As described, iPS-derived cells have a huge potential to model human diseases and to uncover disease mechanisms and novel therapeutic targets. However, their fetal-like and immature nature, as evidenced by the lack of 4R tau expression or only very late expression after one year of culture (Sposito et al., 2015) as well as low levels of certain synaptic proteins (Zhang et al., 2013), is a major limitation. We show increased levels of NCAM1, GluR2, and 4R tau in our 3D culture system compared to those in 2D culture. NCAM1 plays a pivotal role in neuronal maturation and synaptogenesis, and is upregulated during long-term memory formation (Vukojevic et al., 2020). GluR2 has been shown to correlate with neuronal maturation in the hippocampus (Hagihara et al., 2011). While GluR2 is not expressed in neural progenitor cells and only weakly in immature cells, it is strongly expressed in mature neurons *in vivo* (Hagihara et al., 2011). AMPA receptors are also upregulated during synaptic long-term potentiation and memory formation (Tackenberg et al., 2014), agreeing with our finding on NCAM1 upregulation, which suggested increased maturity of iPS-neurons in 3D. As described above, 4R tau has been observed in mature neurons but not in iN cells (Birnbbaum et al., 2018). However, we were able to detect 4R tau in 3D iN cell cultures after 3 weeks of differentiation, which is much earlier than reported in previous studies using iPSC neurons (Choi et al., 2014; Sposito et al., 2015; Miguel et al., 2019), highlighting the potential of this technique in modeling tauopathies such as AD. Nevertheless, it should be noted that our conclusion that neuronal maturation was increased is based on increased expression of maturation markers.

5. Conclusions

Taken together, we describe a simple, sequential seeding-compatible method for 3D cell culture and provide further evidence of increased maturation of iPSC-derived neurons cultured in a 3D microenvironment. Our newly established tool can serve as an easily accessible, fast (3 weeks), and drug-screening-compatible (96-well format-based) platform to identify new pathways of brain diseases and screen for novel or validated current therapeutic targets.

Ethics approval

All experiments were conducted with the approval and according to the regulations of the Cantonal Ethics Committee and informed consent of participants. Animal experiments were performed in compliance with Swiss national guidelines and were approved by the veterinarian office of the Canton of Zurich.

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CRedit authorship contribution statement

Sherida M. de Leeuw: Formal analysis, Visualization, Investigation, Methodology. **Stephanie Davaz:** Formal analysis. **Debora Wanner:** Formal analysis. **Vincent Milleret:** Conceptualization, Visualization, Funding acquisition, Methodology. **Martin Ehrbar:** Conceptualization, Funding acquisition, Methodology. **Anton Gietl:** Conceptualization. **Christian Tackenberg:** Conceptualization, Visualization, Investigation, Methodology, Funding acquisition, Supervision, Manuscript writing.

Competing interests

VM and ME are founders and shareholders of Ectica Technologies AG.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jneumeth.2021.109254.

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